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Advanced Mass Spectrometry Technologies for the Study of Microbial Pathogenesis

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Abstract

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) has been successfully applied to the field of microbial pathogenesis with promising results, principally in diagnostic microbiology to rapidly identify bacteria based on the molecular profiles of small cell populations. Direct profiling of molecules from serum and tissue samples by MALDI MS provides a means to study the pathogen-host interaction and to discover potential markers of infection. Systematic molecular profiling across tissue sections represents a new imaging modality, enabling regiospecific molecular measurements to be made *in situ*, in both two- and three-dimensional analyses. Herein, we briefly summarize work that employs MALDI MS to study the pathogenesis of microbial infection.

Introduction

Hospital and community-acquired infections represent an increasing threat to global public health. Pathogens are rapidly developing resistance to therapeutic intervention, further compounding this threat [1,2]. Therefore, a great need exists for advanced analytical technologies to aid both diagnosis of pathogens in clinical laboratories and the discovery of novel targets for therapeutic intervention in research laboratories. Advanced mass spectrometry technologies can meet this need and aid the ability of clinicians to continue successful treatment of infectious disease.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) is an analytical technology that enables direct analysis of biomolecules. MALDI utilizes a matrix, typically

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a small organic acid with strong ultraviolet absorbance, mixed with analytes to aid desorption and ionization (Figure 1 A)[3]. The resulting gas phase analyte ions are detected and displayed in a spectrum according to their mass-to-charge ratios(m/z), which yield specific molecular signatures within complex samples (Figure 1 B). This label-free technology can be used without *a priori* knowledge of sample composition, allowing for the detection of a variety of analytes, from small molecules to large proteins[4]. Combined with efficient analyte identification strategies and the emergence of online searchable databases[5,6], MALDI MS has been successfully applied to a variety of biological samples, including tissue sections [7], plants [8], insects [9], whole animals [10], and microbial colonies [11]. The ability to analyze such a wide range of systems has led to the use of MALDI MS as a clinical tool, particularly in diagnostic microbiology.

Diagnostic Microbiology

MALDI MS can distinguish molecular fingerprints associated with specific microorganisms, allowing for the rapid identification of infectious agents. MALDI MS presents a means to interrogate intact cells from agar plates or liquid media, leading to robust m/z signatures independent of culture conditions[12]. This has been expanded to include identification of fungi and bacteria that are difficult to culture, such as anaerobic or highly infectious bacteria [13–16]. Advances in sample preparation have improved the reproducibility of MALDI MS measurements from intact cells, making it a promising tool for diagnostic microbiology[11,17,18].

Microbial fingerprinting using MALDI MS can provide accurate determination of spectral peaks specific to both species and genus [19,20]. Initial studies have been compiled into a collection of databases that can be used to classify clinical isolates quickly and accurately [11]. Instrumental set-ups, automated analyses, and database searching platforms tailored to these applications are available from commercial instrument manufacturers, including FDA-approved systems like the MALDI Bio Typer system (Bruker Daltonics) and the Vitek microbial identification system (Biomérieux)[21]. Highly specific databases can be created by users that facilitate the differentiation of pathogenic and nonpathogenic bacterial serotypes[22] as well as particularly virulent strains [23]. Furthermore, comparative analyses of spectral information can be used to determine microbial lineage, which provides a rapid and sensitive tool to diagnose and control outbreaks of pathogenic microbes [24]. Successful implementation of this workflow has been extended from clinical diagnostic laboratories to botany labs for the study of plant pathogens and to the food service industry to study food-spoiling pathogens [25,26].

In addition to rapidly identifying bacteria cultured on media, MALDI MS can be used to identify microbial signatures from complex mixtures, including blood cultures, thus enhancing utility in clinical settings. For example, direct MALDI MS analysis of centrifuged blood culture broths from patients with bacteremia in intensive care units has been utilized to accurately identify microbes from complex, polymicrobial samples. The approach also requires substantially less time than previous diagnostic methods[27]. Such advancements greatly decrease both time and cost associated with microbial identification, leading to more efficient antimicrobial intervention. Integration of MALDI MS technologies into clinical

laboratories therefore has the potential to dramatically affect infectious disease medicine and have a positive impact on human health[28].

MALDI MS Profiling of Infected Tissues to Aid Diagnostics

The ability to rapidly identify MS signatures specific to microorganisms in culture is a major advancement. However, the challenge remains to detect pathogen-specific signals in the context of infection, as simple cultures do not mimic the conditions encountered by microorganisms within the host and provide little insight into pathogenesis and virulence. Due to limited abundance of microbial signals in the presence of host response markers, the detection of these signals from within the host presents analytical challenges. Moreover, identification of disease-specific host response signatures is needed to provide a more complete picture of the host-pathogen interface during infection.

MALDI MS profiling experiments collect a number of discrete spectra from various cell types within samples for comparison. This approach was used in a recent study that searched for serum host-response biomarkers for sepsis in neonates in intensive care units. Sepsis-associated m/z values, including m/z 11,528, identified as a variant of the host inflammatory protein serum amyloid A, provided diagnostic markers which could be detected from serum at birth. Scoring of these biomarkers will allow clinicians to determine which patients should be placed on antimicrobial therapies and has prompted the design of a triage strategy for suspected cases of neonatal sepsis [29].

Another study utilized MALDI MS profiling to study the host response to *Staphylococcus aureus* infected skin wounds over time. Skin lesions infected with *S. aureus* were swabbed into sterile water at various time points post-infection. Both swab samples and wound exudate were analyzed by MALDI MS. Comparison of collected spectra from wound beds and cultured *S. aureus* revealed several matching m/z values. Additional signals were detected from the wound bed thought to belong to mouse defensins and blood, highlighting the ability of MALDI profiling to monitor bacterial signals, host response to infection, and wound healing over time[30]. Such analyses could lead to direct microbial analysis from soft tissue infections and aid in diagnosis of chronic wound infections.

In addition to analyzing spotted tissue homogenates or serum, MALDI MS provides the distinct advantage of *in situ* tissue profiling. This method allows molecular signals to be obtained directly from tissue sections in a spatially-targeted approach, allowing pathologists to focus analyses on histological regions of disease[31]. The histology-directed approach has been applied to the study of bacterial sarcoidosis in snap-frozen human tissue. Targeting only the granulomas, m/z values were observed to correlate with either bacterial infectious foci or control tissue[32]. Histology-directed analyses of large sample sets allows for isolation of signals in a high-throughput manner. Identification of biomarkers using traditional proteomic approaches can validate targets for future study.

MALDI Imaging Mass Spectrometry Applications

MALDI Imaging Mass Spectrometry (IMS) enables the spatial distribution of analytes to be determined by collecting spectra at defined locations across samples[7]. Sophisticated

software processes and displays spectra as heat-maps, which reveal the localization and relative intensities of analytes. MALDI IMS has been used to probe microbial interactions *in vivo*. Complex metabolic interactions between gut microbiota and host tissue have been observed directly from cross-sections of mice[10]. In addition to pathogen and host signals that can be studied *in situ*, MALDI IMS enables to study of other small molecules, including pharmaceutical compounds. A notable example using IMS to study the pathogen-pharmaceutical interaction employed MS/MS methods to monitor the antibiotic moxifloxacin in rabbit lungs infected with *Mycobacterium tuberculosis*[33]. This approach enabled the localization of moxifloxacin with respect to mycobacterial granulomas over the time course of infection (Figure 2). The spatial information provided by IMS revealed that moxifloxacin distribution was not homogeneous within the granuloma, with relatively lower concentrations in granuloma centers coinciding with the sites of infectious foci [33].

IMS has been used to study the host response to bacterial infections, with a focus on the identification and localization of host proteins and molecules involved in metal chelation and nutritional immunity [34]. Metal homeostasis is a critical factor that contributes to both the virulence of the pathogen and the host response to infection [35]. Nutritional immunity studies have combined the ability of MALDI to image proteins and small molecules with laser ablation inductively coupled plasma imaging mass spectrometry (LA-ICP-IMS) to image trace metals. IMS has been used to study iron homeostasis during fungal infection, revealing changes in the spatial distribution of the iron-binding protein hemoglobin alpha and the iron protoporphyrin heme B in the kidneys of mice infected with *Candida albicans*. Linking IMS protein imaging and LA-ICP-MS trace metal imaging provided spatial information regarding iron distribution, which was correlated to the distribution of the iron-binding molecules [36].

Further studies using IMS to monitor nutritional immunity determined that the calcium-, manganese-, and zinc-binding protein calprotectin localized to sites of neutrophil aggregation in the kidneys of *S. aureus* infected mice[34,37]. Calprotectin has also been monitored by IM in murine pulmonary infections caused by *Acinetobacter baumannii*. These experiments demonstrated calprotectin localization to sites of inflammatory cell recruitment within infectious foci, as shown in Figure 3 A[38,39]. Calprotectin accumulation in lungs was found to correlate with bacterial burden (Figure 3B)[39]. MALDI IMS analyses therefore enable study of *in situ* interactions between pathogen and host in regions of infection, resulting in unique insights into the host-pathogen interface.

3D Imaging (3D) Mass Spectrometry

The analytical power of 2DIMS experiments can provide unparalleled information about the pathogen-host interaction. However, multiple sections of the same sample can also be analyzed and computationally recombined to provide a three-dimensional analysis of tissues, further increasing the power of IMS as an investigative tool[40]. 3D IMS has been applied to microbes growing on agar plates to reconstruct microbial secretions occurring on the agar surface as well as beneath the surface. Figure 4 shows a selected ion from *C. albicans* with distinct secretion patterns into the agar, highlighting the ability of 3D IMS to discover unique molecules that would be missed in a standard 2D analysis[41]. Such a 3D IMS

approach was used to successfully analyze *in vitro* interactions of *Pseudomonas aeruginosa* and *C. albicans*, two opportunistic pathogens that have previously been co-isolated from the lungs of cystic fibrosis patients[42].

Integrating MALDI IMS with other analytical technologies, such as magnetic resonance imaging (MRI), can provide high-resolution anatomical information to aid in the reconstruction of 3D analyses. Paired with ambient tissue fixations, like PAX gene tissue stabilization reagent, this work flow has allowed the reconstruction of high resolution, multimodal data sets from whole organs [43,44]. Additionally, MALDI IMS and MRI have been successfully used to analyze the immune response of mice to systemic bacterial infections[45]. Using MRI to track infectious foci within the mouse, whole-body MALDI IMS data showed signals specific to infected regions. Coregistering the information from MALDI IMS and MRI allows for the visualization of the inflammatory response in 3D throughout the entire body of a mouse[45].

These 3D data sets are large and complex, which require more computational power for normalization and interpretation as compared to 2D analyses. Additional advancements have been made in algorithms to better register MALDI data in 3D space, as well as to facilitate data mining and representation[46]. Bioinformatics advancements further the use of 3D IMS as a tool to study pathogens and infection.

Looking to the Future

MALDI MS is a maturing technology; each technological advancement yields potential to enhance the capabilities of the diagnostic laboratory. As the clinical microbial laboratory embraces more advanced analytical technologies, the potential for rapid and accurate diagnostics becomes more realistic. Rapid diagnostics are currently attainable for cultured microorganisms, but signals from pathogens directly from the host specimens are more difficult to determine. The challenges arise from both the relative low abundance of microbial proteins and the small size of most microorganisms.

Current work in the field of MALDI MS instrumentation addresses some of these remaining challenges. Advanced laser optics enables high spatial resolution MALDI MS analyses, including MALDI imaging of single cells [47–49]. New detector systems allow for higher molecular weight proteins to be efficiently detected directly from complex environments, including tissue [50]. Advanced electronics in new mass spectrometers enable rapid acquisition of data, ideal for large 3D imaging data sets [51]. Improvement in sample preparation has focused on the enhanced sensitivity and ionization of selected analytes. For example, ionic matrices have detected signals from mouse blood and urine correlating to masses of cultured bacteria in an *in vivo* model of systemic bacteremia[52]. Such ionic matrices improve the ionization of proteins and reduce spot-to-spot variation, characteristics critical for clinical diagnostics[53]. MALDI-compatible surfaces have been designed to capture bacteria from biological solutions. Bacterial signatures from 10^4 colony forming units (CFU)/mL have been detected from titanium-based chips[54]; graphene magnetic nanosheets decorated with chitosan allow detection of bacterial signatures from 10^2

CFU/mL[55]; and zirconium hydroxide immobilization followed by direct cultivation enable detection of 32 CFU/mL of *Enterococcus faecalis* spiked into milk [56].

In conclusion, the substantial technological progress in MALDI MS solidifies its future in both research and clinical settings. Rapid diagnostic capabilities could make this technology a future point-of-care testing method, guiding the approaches of physicians for antibiotic administration and treatment regimens in an era of increased antibiotic resistance. MALDI MS remains a robust and high throughput discovery tool for novel biomarkers from complex mixtures. Histologically directed analyses and imaging experiments provide spatial information of analytes specifically at the pathogen-host interface. Thus, MALDI IMS is a powerful tool for the future study of pathogenesis, the pathogen-host interaction, and diagnostic microbiology.

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Highlights

- MALDI MS advances diagnostic microbiology through rapid microbial identification
- Histology-directed MS analyses define biomarkers in discrete areas of infected tissue
- MALDI IMS provides spatial distributions of analytes in both two and three-dimensions

Figure 1.

A. Schematic of MALDI ionization process. The sample is first mixed with a matrix. Irradiation by a UV laser initiates the ionization/desorption processes, and newly created ions are guided into a mass spectrometer. Ions produce peaks with unique mass-to-charge ratios, or m/z values, which correlate to their protonated molecular weight. B. An example MALDI mass spectrum taken directly from a mouse kidney.

Figure 2.

IMS of moxifloxacin fragment (m/z 402.2 to 384.2) in rabbit lung infected with *M. tuberculosis* at 1.5 hours post antibiotic administration. IMS reveals unique spatial distribution of moxifloxacin; the drug is not uniformly distributed into infectious foci, annotated with arrows, and does not appear to fully penetrate granulomas into regions where the bacteria are speculated to reside. Adapted with permission from (Prideaux B, Dartois V, Staab D, Weiner DM, Goh A, Via LE, Barry CE, III, Stoeckli M: **High-Sensitivity MALDI-MRM-MS Imaging of Moxifloxacin Distribution in Tuberculosis-Infected Rabbit Lungs and Granulomatous Lesions.** *Analytical Chemistry* 2011, **83**:2112–2118.) Copyright 2011 American Chemical Society.

Figure 3.

Analysis of murine pulmonary infection with *A. baumannii*. A. MALDI IMS analysis of lung tissue from mice at selected time points post-infection. Ions at *m/z* 10,165 and 12,299, belonging to the proteins S100A8 and S100A9, are shown. These proteins form the heterodimeric neutrophil protein calprotectin. B. Bacterial burdens from lung homogenates at various times post-infection. Each circle represents a mouse lung homogenate. Adapted with permission from John Wiley and Sons from (Jessica L. Moore, Kyle W. Becker, Joshua J. Nicklay, Kelli L. Boyd, Eric P. Skaar, Richard M. Caprioli: **Imaging mass spectrometry**

for assessing temporal proteomics: Analysis of calprotectin in *Acinetobacter baumannii* pulmonary infection.*Proteomics*, 2013, in press)

Figure 4.

IMS of the ion at m/z 412 from a colony of *C. albicans* grown on agar A. An overhead 2D IMS analysis of m/z 412 reveals that the ion is not very abundant on the agar surface. B. A 2D IMS analysis of the transected colony reveals the ion at m/z 412 is abundantly secreted into the agar. C. Reconstructed 3D analysis of m/z 412 showcases the power of 3D analysis. Not only is the ion readily detected in the agar, but it also reveals that the distribution is not uniform around the colony. Adapted by permission from Macmillan Publishers Ltd: [The ISME Journal] (Watrous JD, Phelan VV, Hsu C-C, Moree WJ, Duggan BM, Alexandrov T,

Dorrestein PC: **Microbial metabolic exchange in 3D**. *The ISME Journal* 2013, **7**:770–780.), copyright 2013.